Studies on Pectic Enzymes of Microorganisms

Part II. Production of Endo-Polygalacturonase with *Aspergillus saitoi**

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Conditions for the production of endo-polygalacturonase (endo-PG) with *Aspergillus saitoi* IAM 2217 in the submerged culture was examined. This strain was selected as the most potent producer of endo-PG. Endo-PG of this strain was produced in the absence of pectin, but the addition of pectin increased endo-PG activity when inoculated with proliferated mycelia.

As far as examined with a modified Czapek medium (ordinary constituents+pectin and ammonium tartrate), the addition of organic nitrogen sources, such as corn steep liquor, markedly reduced the enzyme producibility. As for the carbon and nitrogen amount in the medium, sucrose: 4%, pectin: 2%, NaNO₃: 1.15%, C/N=10, gave the best result among tested.

INTRODUCTION

In the previous paper,¹⁾ it was reported that molds which specifically produced one of several pectic enzymes were isolated and that a few strains of *Aspergillus saitoi* were the specific potent producers of endo-polygalacturonase (endo-PG).

Endo-PG is known to split the α -1,4-glycosidic linkage of polygalacturonic acid at random.^{2,3)}

On the contrary, the real presence of polymethylgalacturonase (PMG) which is thought to split directly the glycosidic linkage of pectin in hydrolytic way, is very uncertain, although it has been reported that the activity of pectin *trans*-eliminase which directly and eliminatively split the α -1,4-glycosidic linkage

of pectin molecules was actually found in fungal pectinase preparations.^{4,5)}

Endo-PG is thought, therefore, to have the most important role in the enzymic degradation of pectic substances if pectinesterase coexist. Then the authors aimed at the production of endo-PG in the submerged culture with the specific potent producer, *Aspergillus saitoi*, and the purification of this enzyme from the crude enzyme preparation.

Saitô⁶⁾ and Tuttobello et al.⁷⁾ already reported the production of polygalacturonases by *Aspergillus niger* in the submerged culture.

According to the result of Saitô,⁶⁾ endo-PG was constitutive throughout the culture periods. On the contrary, Tuttobello et al.⁷⁾ found that the addition of pectin to the culture medium greatly increased the amount of endo-PG excreted to the medium, although the pro-

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7) E. Tuttobello and P. J. Mill, *Biochem. J.*, **79**, 51 (1961).

duction of endo-PG was observed in the absence of pectic substances.

Aspergillus saitoi is closely related to *Asp. niger*, but this species is defined as the different one from *Asp. niger* in our country.⁸⁾ Both *Asp. niger* and *Asp. saitoi* have black conidia, less than 5 μ in diameter, and cannot assimilate nitrous acid.

The matured conidia of *Asp. saitoi*, however, is characterized with smooth or rough surface in comparison with the matured conidia of *Asp. niger* with conspicuous echinulae or spinules.

It is the purpose of this report to inform the cultural conditions for the production of endo-PG by the submerged culture with the specific potent producer, *Aspergillus saitoi* IAM 2217.*

MATERIALS AND METHODS

Culture medium

Unless otherwise stated, a modified Czapek medium was employed throughout this study, which contained sucrose, pectin N. F.** (Sunkist Growers, Inc., Ontario, Calif., U.S.A.), NaNO₃, 0.5% of ammonium tartrate, 0.1~0.2% of K₂HPO₄, 0.5% of KCl, 0.05% of MgSO₄·7H₂O, trace of FeSO₄, and 0.003% of yeast extracts.

The concentration of sucrose, pectin N. F., NaNO₃ was varied according to experimental purposes and will be mentioned in detail in each experiment.

Culture conditions

Usually the conidia of *Aspergillus saitoi* IAM 2217 were inoculated to a bread medium which was solely composed of autoclaved wet bread. After three or four days' culture at 30°C, the surface of the bread was covered with black conidia, which were collected with pouring sterilized distilled water containing 0.05% of sucrose monostearate. The conidia thus obtained were inoculated to 100 ml of the inoculum medium (sucrose: 3%, pectin N. F.: 0.75%, NaNO₃: 0.58%, C/N=10) contained in the 500 ml shaking flask at the final concentration of 1.0~1.5×10⁶ conidia/ml.

After being cultured for 42~48 hours on a recipro-

cal shaker at 125 oscillation per minute, an amplitude of 7.5 cm, 30°C, 6~7 ml of the developed mycelial suspension was transferred to 100 ml of the main culture medium in 500 ml shaking flasks and cultured at 100 o.p.m., an amplitude of 7.5 cm and 30°C. The concentration of sucrose, pectin N. F., and NaNO₃ of the main culture medium will be mentioned in individual experiment as stated above. The activity of endo-PG in the main culture medium was assayed periodically.

Evaluation of endo-PG activity

Endo-PG activity was measured after Saitô's method by the decrease in viscosity of sodium polypectate (Sunkist Growers Exchange Lot 9649) using an Ostwald viscosimeter.⁹⁾ One viscosity diminishing unit was defined as the amount of enzyme which reduced the viscosity of 1 ml of a 0.8% solution of sodium polypectate by 50% in 10 minutes at pH 4.5 and 40°C.

To 5 ml of 0.8% of sodium polypectate solution which contained final 0.05 M acetate buffer pH 4.5, 0.2 ml of appropriately diluted enzyme solution was added. The reaction mixture was transferred to an Ostwald viscosimeter whose substrate blank and water blank were 90 and 6.5 seconds respectively. Fig. 1 shows the standard curves for endo-PG assay. Per cent viscosity change, expressed in Fig. 1, was calculated from Roboz et al.'s equation.¹⁰⁾

$$\text{Per cent viscosity change} = \frac{V_o - V_t}{V_o - V_s} \times 100 :$$

where

V_o = flow time in seconds of Na-polypectate + inactivated enzyme (substrate blank)

V_t = flow time in seconds at reaction time of Na-polypectate + active enzyme

V_s = flow time in seconds of the solvent + inactivated enzyme (water blank).

Evaluation of endo-PMG activity

Endo-polymethylgalacturonase (endo-PMG) activity was measured in the similar way as done for the endo-PG activity assay using 1.3% pectin solution (containing final 0.05 M acetate buffer pH 3.5 or 4.5) and the same Ostwald viscosimeter.

Simplified evaluation of endo-PG activity

The activity of endo-PG was in part estimated by the disk plate method as previously reported.¹¹⁾ In

8) K. Sakaguchi, H. Iizuka and S. Yamazaki, *Ôyôkingaku*, **3**, 97 (1950).

* Formerly reported as *Asp. saitoi* 4-3.

** N. F.: National Formulary.

9) H. Saitô, *J. Agr. Chem. Soc. Japan*, **28**, 814 (1954).

10) E. R. Roboz, R. W. Barrat and E. Tatum, *J. Biol. Chem.*, **195**, 459 (1952).

FIG. 1. Standard Curves for Endo-PG Activity.
Reaction time is shown in minutes.

this experiment, however, pulp disks (Tôyô Filter Paper Co.), 8 mm in diameter, were used in stead of the disks made of Tôyô filter paper No. 2. The standard curve was prepared by placing the disks moistened with the serial concentration of the enzyme solution of known activity and the activity of endo-PG was expressed in the unit as defined above.

Chromatography of galacturonides

To 5 ml of 1% polygalacturonic acid solution, which was previously adjusted to pH 4.8 with final 0.05 M acetate buffer, 0.2 ml of the culture filtrate was added. The enzyme reaction was carried out at 30°C. Aliquots of 15 μ l of the mixture were withdrawn periodically and applied to a Tôyô filter paper No. 50 (40 $\times$ 40 cm). The paper was ascendingly developed with butanol-acetic acid-water (5:2:3). Aniline phthalate was used as a color reagent.

RESULTS

The selection of potent producers of endo-PG

In the previous paper,¹⁾ 6 strains of mold were isolated as the most potent producers of endo-PG. These strains were further examined for their enzyme producing ability in a shaking culture. Mycelia or conidia were inoculated to 10 ml of the modified Czapek medium mentioned above placed in 20 $\times$ 200 mm test tubes, which were shaken for five days at 30°C. The concentration of sucrose, pectin N.F., and NaNO₃ of the medium were fixed at 2, 0.5 and 0.2% respectively. In this experiment, endo-PG activity at pH 3.5 and endo-PMG activity at pH 3.5 and 4.5 were also measured. *Aspergillus saitoi* 4-3 (IAM 2217), as shown in Table I, had the highest endo-PG producing ability. Thus this strain was further examined for its cultural conditions.

The effect of pectin on the production of endo-PG

In order to know whether the endo-PG of *Asp. saitoi* IAM 2217 is constitutive or induced enzyme, the following experiment was carried out. At first, conidia of *Asp. saitoi*, at 1×10^6 conidia per ml of the medium, were inoculated to the ordinary Czapek medium (sucrose: 2%, K₂HPO₄: 0.1%, NaNO₃: 0.3%, KCl: 0.05%, MgSO₄·7H₂O: 0.05%, FeSO₄: trace) containing 1% of pectin or 1% of sucrose instead of pectin, and shaken at 125 o.p.m. for 2 days at 30°C. Then about 7 ml of the suspension of mycelia of the plus pectin flasks were

TABLE I. COMPARISON OF ENDO-PG PRODUCING ABILITY OF SEVERAL STRAINS OF MOLD IN A MODIFIED CZAPEK MEDIUM

	Endo-PG activity (unit/ml)		Endo-PMG activity (unit/ml)	
	at pH 3.5	at pH 4.5	at pH 3.5	at pH 4.5
<i>Asp. saitoi</i> 4-3 (IAM 2217)	160	1,100	165	650
<i>Asp. saitoi</i> 7-6	79	530	74	300
<i>Pen. islandicum</i> Sopp 777	12	43	21	22
<i>Pen. islandicum</i> Sopp 1270	9	43	21	22
<i>Pen. islandicum</i> Sopp 1212	7	12	8	8
<i>Asp. nakazawai</i> 11-8	12	48	10	16

(after 5 days' shaking culture)

transferred to the same new Czapek medium containing 1% pectin or another 1% of sucrose. The mycelial suspensions from flasks without pectin were also transferred similarly to the new Czapek medium containing pectin or no

pectin.

Then the shaking flasks were shaken at 125 o.p.m., 30°C, and the endo-PG activity was measured periodically by the disk plate method. The result is shown in Fig. 2. The detectable amount of endo-PG was found in the pectin minus flask inoculated with mycelia grown in the pectin minus medium. But the addition of pectin to the medium markedly increased the endo-PG activity.

As shown in Fig. 3, the chromatographic patterns of the enzymic hydrolysates were different between pectin plus lots ((+) $\rightarrow$ (+), and (-) $\rightarrow$ (+)) and pectin minus lots ((-) $\rightarrow$ (-), and (+) $\rightarrow$ (-)).

Comparison between inoculating conidia and mycelia

When conidia of *Asp. saitoi* IAM 2217 were directly inoculated to the modified Czapek medium, not only the effect of pectin on the formation of endo-PG was not observed, but the endo-PG activity in the same experimental lot was fairly fluctuated between flasks.

But this fluctuation was partly overcome by inoculating mycelia instead of conidia. Thus two sorts of the modified Capek media were provided, the one for the inoculum medium,

FIG. 2. The Effect of Pectin on the Production of Endo-PG When Inoculated with Mycelia.

The symbols (+) and (-) stand for pectin containing and minus media respectively, and the symbol $\rightarrow$ stands for a transfer.

FIG. 3. The Chromatographic Patterns of the Enzymic Hydrolysates of Polygalacturonic Acid.

The enzyme solutions, i.e., culture filtrates, were obtained from 6 days' culture after the mycelial suspensions were transferred to the new medium.

The symbols used for the experimental lots are the same ones as defined in Fig. 2.

and the other for the main culture medium. The enzyme activity in the main culture medium was followed periodically after the mycelia proliferated in the inoculum medium were inoculated. As shown in Figs. 4 A and 4 B, the inoculation of mycelia proliferated in the inoculum medium gave fairly higher activity than direct inoculation of conidia.

FIG. 4. Effects of the Type of Inoculation and Organic Nitrogen Sources on the Development of Endo-PG Activity of *Aspergillus saitoi*.

●: control, ○: plus corn steep liquor (2%), ×: plus wheat bran (2%). Only in this experiment (A and B) conidia were collected from the sporulation medium.¹¹⁾ The concentration of sucrose, pectin, and NaNO₃ of the main culture medium was 3, 0.75 and 0.58% respectively (C/N=10).

The effect of organic nitrogen sources

Figs. 4 A and 4 B also show the effect of organic nitrogen sources upon the enzyme producing ability. It may clearly be said that the addition of organic nitrogen sources, such as corn steep liquor, wheat bran markedly reduced the amount of the enzyme in the medium. Another experiment showed the same tendency, when coprameal, defatted soy bean and urea were added as organic nitrogen sources in addition to NaNO₃.

The effect of the amount of carbon source and pectin

It is reasonably considered that the amount of energy or carbon source, i.e., sucrose, and of inducer, i.e., pectin, may affect the yield

FIG. 5. Effects of the Change in Pectin and Sucrose Amount of the Modified Czapek Medium.

(A): sucrose/pectin=2, (B): sucrose/pectin=4
●: sucrose; 4%, ○: sucrose; 3%, ×: sucrose; 2%

of endo-PG. Figs. 5 A and 5 B show the effects of the amount of carbon source and of inducer, when weight ratios of sucrose/pectin were fixed at 2 and 4 respectively. In either case, C/N ratio was 10. The highest activity was observed in the case of sucrose: 4% and pectin: 2% among tested. It should be mentioned here about the low enzyme activity compared with that shown in Fig. 4. Transfer of the strain from a sporulation medium¹¹⁾ to the bread medium to obtain a large amount of conidia between the experiments shown in Figs. 4 and 5 resulted in the decrease of the enzyme producing ability.

The effects of C/N ratio and the presence of ammonium salt

The effects of C/N ratio and the presence

TABLE II. THE EFFECTS OF NH₄-TARTRATE AND C/N RATIO* ON THE PRODUCTION OF ENDO-PG. (4 days' culture)

C/N	Endo-PG activity (unit/ml)	
	(+) NH ₄ -tartrate	(-) NH ₄ -tartrate
7	8,100	7,800
8	7,600	8,400
9	7,200	8,400
10	8,100	7,800

* Sucrose: 4%, pectin: 2%. C/N ratio was varied with NaNO₃.

11) C. Thom and K.B. Raper, "A Manual of the *Aspergilli*", The Williams and Wilkins Co., 1945, p. 37.

of ammonium salt on the enzyme producibility was summarized in Table II. Any remarkable difference was not observed when C/N ratio varied from 7 to 10 in the presence or absence of ammonium tartrate.

The effect of the rate of oscillation

The rate of oscillation which relates to the rotation speed and the degree of aeration in a large scale fermentation, was compared between 125 and 100 o.p.m. The former condition was much more aerobic than the latter. The endo-PG rapidly decreased in amount after four days when shaken 125 o.p.m. The reduced aeration seemed better for the production of endo-PG.

A large scale fermentation

A 300 l scale fermentation in a 400 l fermentor was tried.

For the proliferation of mycelia, the inoculum medium and aerobic conditions were selected. In the final step large scale fermentation, the main culture medium and less aerobic conditions were adopted. The development of the fermentation is shown in Fig. 6. The culture fluid was concentrated by seven times and the enzyme in the medium was

precipitated by the addition of twice volume of methanol at 5°C. About 40% of the activity in the culture fluid was recovered in the precipitates, although a preliminary experiment showed the over 80% recovery through this treatment. Several data concerned the fermentation and the preparation of the crude enzyme were listed in Table III.

TABLE III. THE DATA CONCERNED WITH THE LARGE SCALE FERMENTATION AND THE CRUDE ENZYME PREPARATION

Total unit in the medium	5.4×10^8 unit
Scale of culture	300 l
Maximum activity in the medium	2,200 unit/ml
Specific activity of the crude enzyme	4.85×10^4 unit/g
Amount of the crude enzyme	6.04 kg
Total unit of the crude enzyme	2.92×10^8 unit
Recovery (medium → Precipitates)	41%

By the addition of methanol, undegraded or partly degraded pectin was also precipitated, so that the specific activity of the crude enzyme preparation was low.

DISCUSSION

The endo-PG of *Aspergillus saitoi* IAM 2217 seemed to be constitutive, because detectable amount of endo-PG was produced in the absence of pectic substances. This observation is consistent with the results concerning the endo-PG production with *Asp. niger*.^{6,7)} The stimulative effect of pectin on the production of endo-PG was also observed as reported with *Asp. niger*.⁷⁾

Increased activity of endo-PG, however, may partly depend on the induced synthesis of exo-polygalacturonase as stated by Saitô,⁶⁾ because the crude enzyme obtained from pectin plus lots produced more monomer of galacturonic acid from polygalacturonic acid than from pectin minus lots. (See Fig. 3)

Tuttobello et al.⁷⁾ observed that the addition of 2% of ground nut meal to the medium markedly increased the amount of endo-PG excreted with *Asp. niger*. According to their

FIG. 6. Progress of a large scale (300 l) fermentation with *Asp. saitoi*.

activity: —●—, residual sugar: ---●---
The concentration of sucrose, pectin and NaNO_3 of the main culture medium was 4, 2 and 1.15% respectively (C/N=10).

result, soya flour, corn steep liquor and nutrient broth increased the producibility of endo-PG as well. As for *Asp. saitoi* IAM 2217, on the contrary, organic nitrogen sources, such as corn steep liquor, coprameal and defatted soy bean were all repressive. In recent years, there are several reports on the repressive effect of amino acids on the exocellular enzyme formation.¹²⁻¹⁴⁾ But the effect of single or combination of amino acids has not been examined with *Asp. saitoi*.

As the formation of endo-PG of *Asp. saitoi* usually reached maximum after 4~6 days' shaking culture, the development of endo-PG activity did not accord with the proliferation of mycelia.

It seems better to reduce the proliferation of mycelia from the following facts: (i) The addition of organic nitrogen sources reduced the producibility of endo-PG. (ii) The re-

duced aeration (100 o.p.m.) seemed superior to increased aeration (125 o.p.m.). (iii) The pellet form of mycelia seemed superior to the pulp form, which was thought to be better grown than the pellet form.

Mill and Tuttobello,¹⁵⁾ in their trial in a 10 l jar fermentor, obtained the activity of about 9,000 unit/ml. While with *Asp. saitoi* IAM 2217, about 8,000 unit/ml (in Mill et al.' unit) was usually recorded in the shaking flask, but fairly reduced activity (about 2,000 unit/ml) was recorded in the 300 l tank fermentor.

The rapid proliferation of mycelia in the tank fermentor may have resulted in the low activity.

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15) P. J. Mill and R. Tuttobello, *Biochem. J.*, **79**, 57 (1961).